Full Paper

Synthesis of New 2-Phenyladenines and 2-Phenylpteridines and Biological Evaluation as Adenosine Receptor Ligands

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Synthesis and biological assays of a series of 2-phenylpteridine derivatives are described to compare their affinities to adenosine receptors with those of the corresponding adenines, purposely prepared, and 8-azaadenines previously described. This study demonstrates that the enlargement of the five-membered ring of the adenine nucleus to a six-membered one is a modification that does not allow the molecules to maintain high activity towards adenosine receptors; in fact, pteridine derivatives did not show themselves to be good adenosine receptor ligands. On the contrary, N^6-cycloalkyl- or N^6-alkyl-2-phenyladenines showed a very high affinity and selectivity for A_1 adenosine receptors. We demonstrate also that the 9-benzyl substituent is crucial for conferring high affinity for A_3 receptors to molecules having a 2-phenyladenine-like nucleus.

Keywords: A_1 Adenosine receptors / A_3 Adenosine receptors / 2-Phenyladenines / 2-Phenylpteridines

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Introduction

Adenosine is constitutively present at low levels in the extracellular space but, in metabolically stressful conditions, such as in injury, ischemia and inflammation, its extracellular concentration dramatically increases. Adenosine, acting mostly at its receptors, is a powerful signalling molecule that participates in the regulation of a wide variety of physiological and pathophysiological processes [1].

Four adenosine receptors were identified and cloned: A_1, A_{2A}, A_{2B} and A_3 [1]. A_1 receptors are widely distributed in the central nervous system and in peripheral tissue and mediate diverse biological effects. For example the role of adenosine as a neuroprotective agent during hypoxia and ischemic conditions seems to be mediated by the A_1 receptors; these receptors have been implicated in sedative, anticonvulsant and anxiolytic effects. A_1 receptors mediate cardiac depression through negative chronotropic, dromotropic and inotropic effects. In the kidney, activation of A_1 receptors causes vasoconstriction, inhibition of renine secretion, diuresis, natriuresis, and others effects [1]. Extracellular adenosine and adenosine A_{2A} and A_3 receptors serve as anti-inflammatory signals and sensors of excessive inflammatory tissue damage [2]. A_{2A} receptors play a role in mediating pain via peripheral sites, inhibiting platelet aggregation and regulating blood pressure [3]. A_{2A} receptors are also critically important for the motor stimulant effects of caffeine [3, 4] and regulate the inflammatory response [5]. Adenosine A_3 receptors have shown important roles in physiological systems, including protective actions against myocardial and brain ischemia [6 – 9], antiproliferative effects [10 – 12] and airway inflammation [13, 14] implicating a potential drug target for ischemia, cancer and asthma.

In the past, we have synthesized and assayed a large number of ligands of A_1, A_{2A} and A_3 adenosine receptors, some of these demonstrated a very good affinity and selectivity for A_1 and A_3 subtypes. During our research, we have studied some modifications of the purine nucleus to demonstrate the importance of the kind of atoms present in the various positions of this nucleus. In

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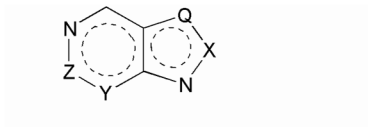


adenine nucleus : X=C; Y=N; Z=C; Q=N
nucleus A : X=N; Y=N; Z=C; Q=N
nucleus B : X=N; Y=C; Z=N; Q=N
nucleus C : X=C; Y=N; Z=C; Q=S
nucleus D : X=N; Y=C; Z=C; Q=N
pteridine nucleus : X=CH=CH; Y=N; Z=C; Q=N

Figure 1. Purine-like nuclei, synthesized as adenosine receptor ligands.

Fig. 1 are shown the purine-like nuclei that have been synthesized and assayed by us. In a recent paper, we demonstrated bioisosterism of the 8-azaadenine (nucleus A, Fig. 1) and adenine nucleus [15]; instead, other modifications (nucleus B, C, D, Fig. 1) caused a decrease of affinity of the compounds with respect to the corresponding adenines. In particular, among all the inactive 1,2,3-triazolo[4,5-d]pyridazines (nucleus B, Fig. 1) [16–18] synthesized, only the 3-benzyl-4-(*m*-tolylamino)-1,2,3-triazolo[4,5-*d*]pyridazine-7-one showed good affinity towards A_1 receptors [18] and no 3-benzyl-5-phenyl-7-alkylaminothiazolo[4,5-d]pyrimidine-2(*3H*)-thiones [19] (nucleus C, Fig. 1) were a good ligand. Regarding 3-deaza-8-azaadenines (nucleus D, Fig. 1), this nucleus can be considered bioisoster of adenine, but also this modification caused a general lowering of affinity with respect to the corresponding adenines and 8-azaadenines [20].

The pteridine nucleus can also be viewed as a modification of the adenine nucleus: the carbon atom in position 8 is substituted by an ethylene group (see Fig. 1). In the past, other authors also have considered pteridines structurally similar to purines [21], so we decided to evaluate how this modification could affect the activity of some active adenosine receptor ligands synthesized now or in the past.

The pteridine nucleus is a very important bicyclic nucleus present in many natural compounds and is studied from the chemical and biological points of view. The natural pteridine pigments can be regarded as the origin of pteridine chemistry since, more than 100 years ago, Hopkins focused his attention for the first time on these compounds [22] on English Brimstone butterfly pigments (Fig. 2). The components were isolated and structurally identified by Purrmann [23] in 1940 and the new ring system was termed pteridine (Fig. 3) by Wieland [24]. Another group of natural pteridine pigments is present in the eyes of *Drosophila melanogaster* and in the dorsal skin of frogs. More natural substances contain the pteridine nucleus in their molecular structure, such as folic acid, riboflavine, and the cofactor of some enzymes *e. g.*

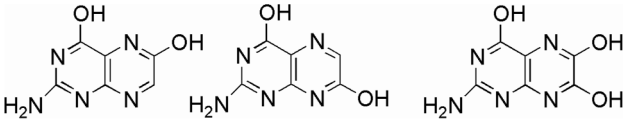
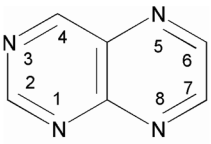


Figure 2. Structure of Brimstone butterfly pigments.



Pteridine

Figure 3. Pteridine ring system.

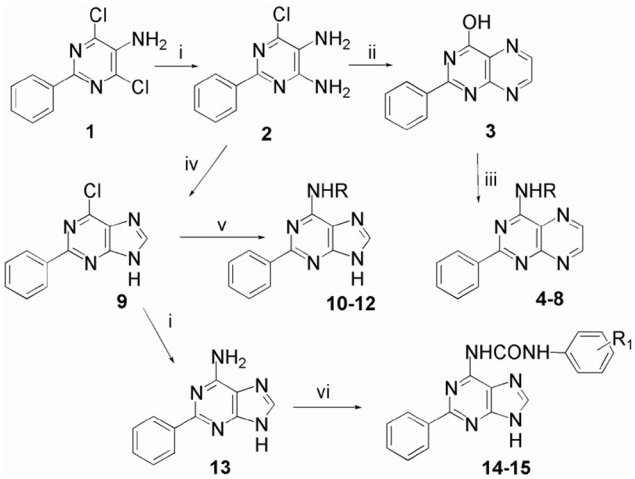
nitroxide synthase, dimethylsulfoxide reductase or xanthine oxidase [25].

In this paper we describe the synthesis and biological assays of a series of 2-phenylpteridine derivatives, we compare their affinities toward adenosine receptors with those of the corresponding adenines, purposely prepared, and 8-azaadenines previously described.

Results and Discussion

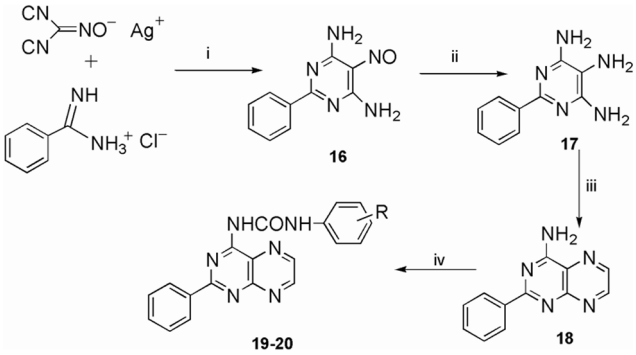
Chemistry

Compounds **4–8, 10–12** and **14–15** were synthesized starting from 4,5-diamino-6-chloro-2-phenylpyrimidine **2** [15] (Scheme 1), obtained from 5-amino-4,6-dichloro-2-



Reagents: i: NH_3, 110°C, 24 h; ii: Glyoxal, EtOH/AcOH/H_2O, reflux, 1 h; iii: Suitable amine, HMDS, 110°C, 6 h; iv: Triethylorthoformate, acetic anhydride, 120°C, 90 min; v: Suitable amine, absolute ethanol, 90°C, 9 h; vi: Suitable isocyanate, CH_3CN, reflux, 3 h.

Scheme 1. Synthetic pathway of compounds **10–12** and **14–15**.



Reagents: i: stirring 1 h, then 2-methylpyridine, reflux, 30 min; ii: THF, $Na_2S_2O_4$, NaOH ; iii: glyoxal, EtOH/AcOH/H_2O, reflux, 1 h; iv: suitable isocyanate, CH_3CN, reflux, 3 h.

Scheme 2. Synthetic pathway of compounds **19 – 20**.

phenylpyrimidine **1** [26] by a known synthetic pathway [15]. The diamino derivative **2** was cyclised with glyoxal (40 wt.% solution in water) in ethanol and glacial acetic acid: the compound formed was 2-phenyl-4-hydroxy-pteridine **3** [27] owing to displacement of the chlorine atom by a hydroxyl group. Treatment of **3** with 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and the suitable amine in the presence of ammonium sulphate as a Lewis catalyst, gave compounds **4 – 8**.

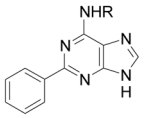
The chloro-purine **9** was obtained by cyclisation of the diamino derivative **2** with triethylorthoformate and acetic anhydride at reflux temperature as reported in the literature [15]. Displacement of the chlorine atom of **9** with suitable amines in absolute ethanol gave derivatives **10 – 12**; the same reaction with gaseous ammonia in absolute ethanol gave 2-phenyladenine **13** [28], which was converted in the 2-phenyl-N^6-(substituted)-phenylcarbamoyladenines **14** and **15** by treatment with the corresponding isocyanates and triethylamine in fresh distilled THF at reflux temperature.

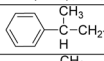
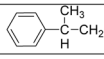
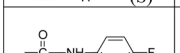
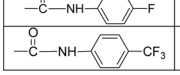
A faster route (Scheme 2) was used to obtain the phenylureidopteridine derivatives **19** and **20**. The reaction of benzamidine chloride with a slight excess of silver salt of isonitrosomalononitrile gave 4,6-diamino-5-nitroso-2-phenylpyrimidine **16** as described in the literature [29, 30]. Compound **16** was treated with sodium hydrosulphite to give, by reduction of the nitroso group, 4,5,6-triamino-2-phenylpyrimidine **17** [30, 31]. Cyclisation of **17** with aqueous glyoxal furnished **18** [31] which reacted with the suitable isocyanates to give the 1-substituted phenyl-3-(2-phenyl-pteridin-4-yl)-ureas **19** and **20**.

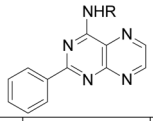
Biological assays

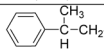
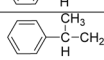
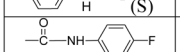
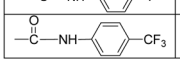
All new compounds obtained were tested in radioligand binding assays for affinity towards A_1, A_{2A}, and A_3 adenosine receptors. The results are reported in Table 1; when

Table 1. Biological results. The K_i values are the mean ± SEM of four separate assays, each performed in triplicate.



R	Compound	A_1 K_i (nM) or I (%)[§]	A_{2A} K_i (nM) or I (%)[‡]	A_3 I (%)[#]
	**10**	5.6 ± 0.8	84.1 ± 5.8	<60%
	**11**	13.6 ± 1.2	1500 ± 120	<60%
$(CH_2)_3CH_3$	**12**	9.6 ± 1.0	700 ± 80	<60%
	**I**[15]	12.8 [15]	<60% [15]	Not assayed
	**II**[15]	297 [15]	<60% [15]	Not assayed
	**14**	78% 1 μM	< 60%	<60%
	**15**	65% 1 μM	<60%	<60%

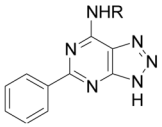


R	Compound	A_1 K_i (nM) or I (%)[§]	A_{2A} I (%)[‡]	A_3 I (%)[#]
	**4**	66 ± 5	<60%	<60%
	**5**	920 ± 10	<60%	<60%
$(CH_2)_3CH_3$	**6**	520 ± 48	<60%	<60%
	**7**	1240 ± 90	<60%	<60%
	**8**	<60% 10 μM	<60%	<60%
	**19**	<60% 10 μM	<60%	<60%
	**20**	<60% 10 μM	<60%	<60%

the percentage of inhibition resulted <60% at 10 μM, compounds were considered inactive.

Adenine derivatives **10 – 12** showed a very high affinity towards A_1 receptors and much lower or no affinity for A_{2A} and A_3, so they can be considered selective for A_1 subtype; also compound **10**, showing an interesting K_i value (84.1 nM) for A_{2A} receptors, has a selectivity A_{2A}/A_1 of 15. These compounds were more active than the corresponding 8-azaadenine derivatives synthesized and assayed in the past [15, 32] (see compounds **III-V** in Table 2). Among pteridine derivatives, only compound **4** showed good activity towards A_1 receptors (K_i = 66 ± 5 nM), compounds **5 – 7** showed low activity (in the range of micromolar), and compound **8** was completely inactive for all the receptor subtypes. These results demonstrated that the activity of pteridine compounds towards A_1 receptors is dramatically lower with respect to the corresponding adenine derivatives (**10 – 12, I, II**) and also of the corre-

Table 2. N^6-substituted-2-phenyladenines synthesised and assayed in the past.



R	Compound	A_1 K_i (nM)	A_{2A} I (%) (10 μM)
	**III** [32]	19.1 [32]	< 60% [32]
	**IV** [32]	76 [32]	< 60% [32]
$(CH_2)_3CH_3$	**V** [32]	134 [32]	< 60% [32]
 (R)	**VI** [15]	354 [15]	< 60% [15]
 (S)	**VII** [15]	4620 [15]	< 60% [15]

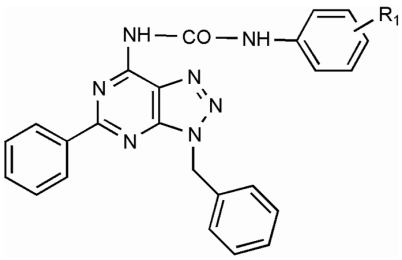


Figure 4. Chemical structure of active 8-azaadenines

sponding 8-azaadenine derivatives (Table 2, compounds **III**–**VII**).

Compounds **14**, **15**, **19** and **20** were synthesized and assayed on the basis of results recently obtained regarding the high affinity of some 9-benzyl-2-phenyl-N^6-substituted-phenyl-carbamoyl-8-azaadenines for A_3 adenosine receptors (Fig. 4) [33]. Regarding compounds **14** and **15**, we observed that the insertion of a phenylureic function on the 6-position of 2-phenylpurine leads to molecules that bind adenosine receptors very weakly. The main structural difference between these molecules and the active 8-azaadenines shown in Fig. 4 is the absence of the benzyl group in the 9-position; this fact should be the reason for the lack of activity of **14** and **15**. Also in the case of compounds **19** and **20**, we can see low percentages of inhibition. For these pteridine derivatives the low activity could be due to two reasons: the absence of the substituent in the 8-position of 2-phenylpteridine (corresponding to the 9-position of 8-azaadenines) and the change of the nucleus structure, from 8-azaadenine to pteridine.

Conclusions

This study demonstrates that, as pteridine derivatives cannot be considered good ligands of adenosine receptors, the enlargement of the five-membered ring of adenine nucleus to a six-membered one is a modification that does not allow the molecules to maintain high activity towards these receptors. On the contrary N^6-cycloalkyl- or N^6-alkyl-2-phenyladenines showed a very high affinity and selectivity for A_1 adenosine receptors. The affinity values are comparable with those of the 9-benzylsubstituted analogous ones (N^6-cyclopentyl-9-benzyl-2-phenyladenine; K_i value of 12.2 nM, N^6-cyclohexyl-9-benzyl-2-phenyladenine; K_i value of 9.4 nM) [15], demonstrating that the 9-benzyl substituent is not very important for the binding of these molecules to A_1 receptors. On the contrary, comparing biological results of compounds **14** and **15** with the active compounds synthesized in the past (see Fig. 4) [33], we demonstrate that the 9-benzyl substituent is crucial to confer high affinity for A_3 receptors to molecules having a 2-phenyladenine-like nucleus.

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Experimental

Chemistry

Melting points were determined on a Kofler hot-stage apparatus (C. Reichert, Vienna, Austria) and are uncorrected. IR spectra in Nujol mulls were recorded on a Mattson Genesis series FTIR spectrometer (Mattson Instruments, USA). ^{1}H-NMR spectra were recorded on a Bruker AC 200 spectrometer (Bruker Bioscience, Billerica, MA, USA) in δ units from TMS as an internal standard; the compounds were dissolved in DMSO-d_6. Mass spectra data were obtained with a Hewlett-Packard GC/MS system 5988 (Hewlett Packard, Palo Alto, CA, USA). TLC was performed on precoated silica gel F_{254} plates (Merck, Darmstadt, Germany). Microanalyses (C H N) were carried out on a Carlo Erba elemental analyser (Model 1106; Carlo Erba, Milan, Italy) and were within ± 0.4% of the theoretical values.

*4,5-Diamino-6-chloro-2-phenylpyrimidine **2***

Starting from **1** [26], compound **2** was obtained as described in the literature [15].

*2-Phenyl-4-hydroxypteridine **3***

A mixture of **2** (0.50 g, 2.27 mmol), glyoxal (40 wt.% solution in water) (5 mL), ethanol (5 mL), water (2.5 mL), and glacial acetic acid (1.5 mL) was stirred at reflux for 1 h. After cooling, the mixture was diluted with water and extracted with chloroform. The organic layer was dried over $MgSO_4$, filtered and evaporated to yield a crude product, which was crystallized from ethanol to give **3** [27] (0.256 g, 50.5%) as a white solid: m. p. 280 – 281°C; ^{1}H-NMR: δ = 13.08 (s, 1H, exch), 9.05 (d, *J* = 2 Hz, 1H, arom), 8.82 (d, *J* = 2 Hz, 1H, arom), 8.22 (m, 2H, arom), 7.62 (m, 3H, arom).

*General procedure to obtain compounds **4–8***

A mixture of **3**, (0.250 g, 1.12 mmol), HMDS (0.538 g, 3.36 mmol), ammonium sulphate (5 mg) and the suitable amine (4.0 mmol) was heated at 100°C for 6 h in a steel bomb; after cooling, the mixture was diluted with methanol, evaporated at reduced pressure to yield a crude product that was dissolved in HCl 10% (25 mL) and extracted with chloroform. The organic layer was dried over $MgSO_4$, filtered, evaporated and the residue was flash-chromatographed on silica gel using ethyl ether/petroleum ether 40 – 60° (85 : 15).

*4-Cyclopentylamino-2-phenylpteridine **4***

0.152 g, 46%. ^{1}H-NMR: δ = 9.08 (d, *J* = 2 Hz, 1H, arom), 8.85 (d, *J* = 2 Hz, 1H, arom), 8.40 (m, 2H, arom), 7.62 (m, 3H, arom), 4.73 (m, 1 H, exch), 4.38 (m, 1H, NH-C*H*), 1.56 (m, 8H, $(CH_2)_4$).

*4-Cyclohexylamino-2-phenylpteridine **5***

0.227 g; 66.5%: m. p. 145–147°C; ^{1}H-NMR: δ = 9.08 (d, *J* = 2 Hz, 1H, arom), 8.87 (d, *J* = 2 Hz, 1H, arom), 8.40 (m, 2H, arom), 7.62 (m, 3H, arom), 4.73 (m, 1 H, exch), 4.38 (m, 1H, C-H), 1.56 (m, 10H, $(CH_2)_5$).

*4-n-Butylamino-2-phenylpteridine **6***

0.162 g, 52%: ^{1}H-NMR: δ = 9.08 (d, *J* = 2 Hz, 1H, arom), 8.78 (d, *J* = 2 Hz, 1H, arom), 8.51 (m, 2H, arom), 7.56 (m, 3H, arom), 3.71 (m, 3H, N-CH_2 + exch), 1.72 (m, 2H, CH_2), 1.39 (m, 2H, CH_2), 0.952 (m, 3H, CH_3).

*4-[(R)-(1-Phenyl-ethyl)]amino-2-phenyl-pteridine **7***

0.194 g, 53%: ^{1}H-NMR: δ = 9.28 (d, *J* = 7.8 Hz, 1H, exch), 9.08 (d, *J* = 2 Hz, 1H, arom), 8.80 (d, *J* = 2 Hz, 1H, arom), 8.47 (m, 2H, arom), 7.41 (m, 8H, arom), 5.71 (m, 1H, C*H*-CH_3), 1.69 (d, *J* = 6.8 Hz, 3H, CH_3).

*4-[(S)-(1-Phenyl-ethyl)]amino-2-phenyl-pteridine **8***

0.274 g, 75%: ^{1}H-NMR: δ = 9.33 (d, *J* = 7.8 Hz, 1H, exch), 9.09 (d, *J* = 2 Hz, 1H, arom), 8.82 (d, *J* = 2 Hz, 1H, arom), 8.51 (m, 2H, arom), 7.41 (m, 8H, arom), 5.72 (m, 1H, C*H*-CH_3), 1.69 (d, *J* = 6.8 Hz, 3H, CH_3).

*6-Chloro-2-phenylpurine **9***

Starting from **2**, compound **9** was obtained as described in the literature [15].

*N^6-Cyclopentyl-2-phenyladenine **10***

A mixture of **9** (0.100 g, 0.43 mmol) and cyclopentylamine (0.53 g, 6.2 mmol) in absolute ethanol (3 mL) was heated at 90°C for 12 h in a well-stopped pyrex tube. After cooling, the solvent was evaporated, chloroform was added (10 mL), the solution was washed with HCl 10% and then evaporated to give **10** (0.093 g, 78%) as a white solid: m. p. 238 – 240°C; ^{1}H-NMR: δ = 9.17 (br s, 1H, exch), 8.85 (s, 1H, arom), 8.33 (m, 2H, arom), 7.55 (m, 3H, arom), 4.70 (m, 2H, NH-C*H* + exch), 1.89 (m, 8H, $(CH_2)_4$).

*N^6-Cyclohexyl-2-phenyladenine **11***

was synthesized from **9** (0.100 g, 0.43 mmol) using the procedure described for **4** and was obtained as a white solid (0.065 g, 52%): m. p. 235 – 236°C; ^{1}H-NMR: δ = 8.82 (m, 2H, arom + exch), 8.31 (m, 2H, arom), 7.57 (m, 3H, arom), 4.27 (m, 2H, NH-C*H* + exch), 1.68 (m, 10H, $(CH_2)_5$).

*N^6-n-Butyl-2-phenyladenine **12***

was synthesized from **9** (0.100 g, 0.43 mmol) using the procedure described for **4** and was obtained as a white solid (0.040 g, 35%): m. p. 222 – 223°C; ^{1}H-NMR: δ = 12.92 (s, 1H, exch), 8.36 (m, 2H, arom), 8.08 (s, 1H, C-H), 7.71 (m, 1H, exch), 7.45 (m, 3H, arom), 3.63 (m, 2H, NH-CH_2), 1.67 (m, 2H, CH_2), 1.41 (m, 2H, CH_2), 0.92 (m, 3H, CH_3).

*2-Phenyladenine **13***

Starting from **9**, compound **13** was obtained as described in the literature [28].

*N^6-(4-Fluorophenylcarbamoyl)-2-phenyladenine **14***

To a solution of **13** (0.040 g, 0.119 mmol) and triethylamine (0.019 g, 0.11 mmol) was added dropwise 4-fluorophenylisocyanate (0.019 g, 0.14 mmol) and heated at reflux under stirring for 1 h. After cooling, the solid precipitate was filtered and crystallized from ethanol to give **14** as a white solid: m. p. 196 – 197°C; ^{1}H-NMR: δ = 10.92 (s, 1H, exch), 10.28 (s, 1H, exch), 8.67 (s, 1H, arom), 8.38 (m, 2H, arom), 7.31 (m, 8H, arom + exch). MS: m/z 348 [M$^+$].

*N^6-(4-Trifluoromethylphenylcarbamoyl)-2-phenyladenine **15***

was synthesized from **13** (0.040 g, 0.10 mmol) using the procedure described for **14** to give pure **15** (0.020 g, 54.5%) as a white solid: m. p. 198 – 199°C; ^{1}H-NMR: δ = 11.09 (s, 1H, exch), 10.51 (s, 1H, exch), 8.68 (s, 1H, arom), 8.38 (m, 2H, arom), 7.47 (m, 8H, arom + exch). MS: m/z 398 [M$^+$].

*4,6-diamino-5-nitroso-2-phenylpyrimidine **16***

Compound **16** was obtained as described in the literature [29].

*4,5,6-Triamino-2-phenylpyrimidine **17***

To an iced and stirred suspension of 1.07 g (5 mmol) of **16** in 10 mL of THF, a solution of $Na_2S_2O_4$ dihydrate (3 g) in 20 mL of 1M NaOH was added in small portion (the temperature of reaction must remain under 30°C), then the stirring was maintained for 20 h. The organic phase was separated and evaporated at reduced pressure to give pure **17** (0.8 g, 80%).

*2-Phenylpteridine **18***

Compound **18** was obtained from **16** as described in the literature [31].

*4-(4-Fluorophenylureido)-2-phenylpteridine **19***

was synthesized from **18** (0.040 g, 0.19 mmol), using the procedure described for **14**, to give pure **19** as a with solid (0.056 g, 82%): m. p. 219-220°C; ^{1}H-NMR: δ = 10.99 (s, 1H, exch), 10.26 (s, 1H, N-H), 9.28 (d, J = 1.8 Hz, 1H, arom), 9.00 (d, J = 1.8 Hz, 1H, arom), 8.52 (m, 2H, arom), 7.65 (m, 5H, arom), 7.23 (m, 2H, arom).

4-(4-Trifluoromethylphenylureido)-2-phenylpteridine ***20***
was synthesized from **18** (0.040 g, 0.19 mmol), using the procedure described for **14**, and was obtained as a white solid (0.045 g, 56%): m. p. 226–227°C; ^{1}H-NMR: δ = 11.14 (s, 1H, exch), 10.45 (s, 1H, exch), 9.29 (d, J = 1.7 Hz, 1H, arom), 9.01 (d, J = 1.7 Hz, 1H, arom), 8.56 (m, 2H, arom), 7.75 (m, 7H, arom).

Biological assays

[^{125}I]-ABMECA were purchased from Amersham Biosciences, [^{3}H]CHA and [^{3}H]CGS 21680 were obtained from NEN Life Science Products, Inc. (Köln, Germany). Adenosine deaminase (ADA) was obtained from Boehringer-Mannheim (Mannheim, Germany). Cell culture media and fetal calf serum were obtained from Bio-Wittaker (Walkersville, MD, USA). (-)-N^6-(2-phenylisopropyl) adenosine (R-PIA), and other agents were purchased from Sigma-Aldrich, srl (Milan, Italy).

A_1 receptor-binding assay

Bovine cerebral cortex was homogenised in ice-cold 0.32 M sucrose-containing protease inhibitors, as previously described [34]. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C and the supernatant again centrifuged at 48 000 *g* for 15 min at 4°C. The final pellet was dispersed in 50 mM Tris-HCl, pH 7.7 fresh buffer, incubated with adenosine deaminase (2 units mL^{-1}) to remove endogenous adenosine at 37°C for 60 min, and then recentrifuged at 48 000 *g* for 15 min at 4°C. The pellet was suspended in buffer and used in the binding assay. The [^{3}H]CHA binding assay was performed in triplicate by incubating aliquots of the membrane fraction (0.2–0.3 mg of protein) at 25°C for 45 min in 50 mM Tris-HCl, pH 7.7, containing 2 mM $MgCl_2$, with approximately 1.2 nM [^{3}H]CHA (Kd 0.5 nM). Nonspecific binding was defined in the presence of 50 mM R-PIA. Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure. Filters were washed three times with 5 mL of ice-cold buffer and placed in scintillation vials. Radioactivity was counted in a 4 mL Beckman Ready-Protein scintillation cocktail (Beckman Ready Protein, Fullerton, CA, USA) in a liquid scintillation counter.

A_{2A} receptor binding assay

Bovine striatum was homogenised in 20 volumes of ice-cold 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and protease inhibitors. The membrane homogenate was centrifuged at 48 000 *g* for 10 min at 4°C. The resulting pellet was resuspended in buffer containing 2 units/mL of adenosine deaminase and incubated at 37°C for 30 min. The membrane homogenate was centrifuged, and the final pellet frozen at −80°C. Routine assays were performed in triplicate by incubation of an aliquot of striatal membranes (0.2–0.3 mg of protein) in cold 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ with approximately 5 nM [^{3}H]CGS 21680 (Kd 16 nM) in a final volume of 0.5 mL. Incubation was carried out for 90 min at 25°C. Non-specific binding was defined in the presence of 50 μM CGS 21680. Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure. Filters were washed three times with 5 mL of ice-cold buffer and placed in scintillation vials. Radioactivity was counted in a 4 mL Beckman Ready-Protein scintillation cocktail in a scintillation counter.

A_3 adenosine receptor binding assay

CHO cells, stably transfected by Dr K. N. Klotz with human A_3 adenosine receptors were maintained in Dulbecco's modified Eagle's medium with F12 nutrient mixture without nucleotides, supplemented with 10% fetal calf serum, L-glutamine (2 nM), penicillin (100 units/mL), streptomycin (100 μg/mL) in a humidified athmosphere of 5% CO_2/95% air at 37°C, essentially as previously described [35]. Cell cultures were split two or three times a week in a ratio between 1:5 and 1:20 and used for binding experiments at subconfluency.

At removal, the cells were harvested by centrifugation at 500 *g*. The crude membranes were prepared as described by Olah *et al*. [36]. [^{125}I]AB-MECA binding assay to transfected cell membranes was carried out as previously described [36]. In brief, the cell membranes (80 μg per assay) were incubated in 100 μL of 50 mM Tris, 10 mM $MgCl_2$ and 1 mM EDTA (pH 8.12) which contained [^{125}I]AB-MECA at a concentration of 1 nM (Kd 0.6 nM) and adenosine deaminase (2 units/mL). Non-specific binding was defined by 50 μM R-PIA. Assays were incubated to equilibrium for 1 h at 25°C and rapidly filtered on Whatman GF/C filters.

In all competition experiments the compounds were routinely dissolved in DMSO and added to the assay mixture, final DMSO concentrations never exceeded 1%. At least six different concentrations spanning three orders of magnitude, adjusted approximately for IC_{50} of each compound, were used. IC_{50} values, computer-generated using a nonlinear formula on a computer program (GraphPad, San Diego, CA, USA), were converted to K_i values, knowing the K_d values of radioligands and using the Cheng and Prusoff equation [37].

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